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Aflatoxin Formation on Selected Human Foods by Strains of Aspergillus Flavus, Link, and Penicillium Rubrum, Stroll

Ronald Arlyn Sonstegard

AFLATOXIN FORMATION ON SELECTED HUMAN FOODS BY STRAINS OF *ASPERGILLUS FLAVUS*, LINK, AND *PENICILLIUM RUBRUM*, STOLL

BY

RONALD ARLYN SONSTEGARD

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor / Date

Head, Bacteriology Department Date

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INTRODUCTION

The occasional contamination of foods by biologically active compounds arising from metabolic processes of mold contamination represents a situation which has possibly existed since prehistoric times. This is due in part to the ubiquitous distribution of fungi and the frequent opportunity for their growth during the harvest and subsequent storage of foods, and the recognized capabilities of these organisms to produce complex molecules.

Within the last decade an increasing body of knowledge has become available concerning the effects of various deleterious biological metabolites on the health of animals and man. Increased sophistication of methods and approaches to biological problems and clearer concepts of mechanisms of action at the molecular level have greatly increased our current capabilities of detection, through sensitive bioassay procedures, of individual cellular responses to specific micro insults. Today, more than one thousand toxic fungal products or mycotoxins have been recognized (Hesseltine, 1965).

An increased awareness of toxic fungi dates from 1960 when more than 100,000 turkeys died in England after eating moldy Brazilian groundnut (peanut) meal. Exhaustive investigations that followed revealed that the toxic groundnut meal contained compounds which exhibited a blue fluorescence under ultraviolet light in which fluorescence paralleled the toxicity of the groundnut meal. Investigation of this toxic fluorescent compound indicated that strains of a commonly occurring mold, Aspergillus flavus, were responsible for the production

of the toxin. From this point on, work on the aflatoxins, as the toxic factors were named as a contraction of "A. flavus toxin," has proceeded at a rapid rate. Aflatoxins are recognized today as being among the most carcinogenic compounds known, since trout and mice have developed hepatomas with less than 0.5 parts per billion of aflatoxins in their diet (Sinnhuber, 1965).

Aspergillus flavus, with its associated mycotoxins, is but one of many fungal contaminants which may occur in cereal products or other foods. Estimates of the probable number of species of fungi range from 50,000 to 200,000 species (Emmons et al. 1964). More than 10 per cent of the isolates of common molds are toxigenic (Hesseltine, 1966). Fungi and mycotoxins other than those of A. flavus are now receiving intensive consideration since they are being found in a broad spectrum of human and animal foods.

A more clear understanding of the mechanisms of production of mycotoxins by microbial contaminants in food and their action will have to wait until further studies have been made on the isolation and identification of the molds, their toxins and the evaluation of their biological responses. A major advancement has been in the isolation and structural elucidation of the aflatoxins. It can be anticipated that other compounds of similar character are to be discovered after further study.

Huge industrial enterprises including those producing antibiotics and food enzymes are founded on fungus-based chemistry. The discovery of toxicity in moldy groundnut meal may change our attitudes toward the

toxicity of the commercial fungal metabolites. While many fungi presumably are not harmful, the striking effect of products of one of these, the aflatoxins, has stimulated speculation as to the possible role of other mycotoxins which may affect human and animal health through toxicity or induction of cancer.

The fungi that commonly contaminate and produce their metabolites in foods have not been extensively studied with respect to their occurrence or their concentration in the diet of man. Undoubtedly, with some of the conditions of storage of foodstuffs for domestic animals, fungal contamination and growth are not controlled. Selection and control of food for man is obviously more rigid, but certainly fungal metabolites must develop in these foods from which toxic molds have been isolated. Allcroft and Carnaghan (1963b) noted that aflatoxin was present in milk of cows fed toxic groundnut meal. Thus certain food products are potential sources of mycotoxins.

Mycotoxicosis is a problem which has world-wide significance in terms of public health, agriculture, and economics. To what degree the various metabolites from fungi represent a toxicological and carcinogenic hazard to man has not been fully explored.

To date very little research has been reported on the public health hazard caused by contamination of certain manufactured or prepared human foods by mycotoxin producing strains of commonly occurring fungi.

The purposes of this study were (1) to investigate and demonstrate whether detectable amounts of mycotoxins can be produced by known toxigenic strains of fungi when they were grown on a variety of

manufactured foods such as bread, cheese, jelly and other foods; (2) to determine what types of human foods were suitable substrates for toxin formation; (3) to determine what physiological conditions such as temperature, pH and available water would permit maximum production of mycotoxins; and (4) to develop a rapid method of detection and analysis of aflatoxins in human foods.

It is hoped that this work will add to the existing body of knowledge on the subject, and possibly to contribute to the evaluation of this toxicological and carcinogenic hazard so that commercial and domestic foods for man, which are already in short supply, can be safeguarded in the future.

REVIEW OF LITERATURE

Discovery of Aflatoxins

In 1960, poultry farms in the United Kingdom suffered severe losses of young turkeys and ducks when 100,000 young turkeys died in a few months. Since the cause of the outbreak could not be recognized, it was thought to be a new disease and named Turkey 'X' disease (Blount, 1961). Affected birds died within a week, during which time they lost their appetite, became markedly lethargic, and developed a weakness of the wings. A characteristic attitude of head, neck, and legs was adopted at the time of death. Post-mortem examination showed hemorrhages or pale necrotic lesions in the liver and, frequently, engorged kidneys.

No evidence could be found that Turkey 'X' disease was infectious so the possibility of poisoning was considered. The feed of affected birds was examined thoroughly for bacterial toxins, inorganic poisons, possible organic contaminants such as insecticides, and for plants or plant parts of a poisonous nature. None of these could, however, be detected. It was then realized that a common factor in the outbreaks was the presence in the feed of material from a consignment of groundnut meal from Brazil, the first to have been imported into the United Kingdom from that country. Feeding experiments readily confirmed that toxic meal was the cause. Samples of this consignment of groundnut meal were re-examined for contaminants of the kind referred to above, but none were found. Therefore, it was necessary to study the groundnut meal further to determine the cause of toxicity.

A ten-thousandfold concentration of the toxic activity was achieved by more or less conventional chemical methods for concentrating unknown natural products. It was then observed that the intensity of a characteristic blue fluorescence exhibited by the concentrates in ultraviolet light paralleled the toxicity of the groundnut meal. This led to the development of a chemical method for determining the presence and approximate concentration of the toxic factor (Sargeant et al. 1961a).

With the help of this method many more samples could be tested. It became practicable to start examination of groundnuts and groundnut products from various parts of the world. Subsequently, many samples of groundnut meals were tested for toxicity, with the result that samples from at least 13 producing countries were found to be contaminated with the toxins (Allcroft and Carnaghan, 1963a).

From these tests it soon became apparent that the toxic factor, far from being confined to certain batches of groundnut meal from Brazil, was present in some of the samples obtained from each of the other producing countries. Additional support for this widespread distribution was simultaneously afforded by the diagnosis of further outbreaks of Turkey 'X' disease caused by batches of groundnut meal from countries other than Brazil (Spensley, 1963).

The widespread distribution of the toxic factor, coupled with the fact that only a portion of samples exhibited toxicity, suggested that contamination by a microorganism might be the cause. Sargeant et al. (1961a) associated the toxicity with heavy mold infestation of toxic groundnut meal. He grew some of the fungal species on synthetic media

and on heat sterilized nontoxic groundnut meal and demonstrated the presence of a toxin producing fungal strain identified as Aspergillus flavus. The generic name "aflatoxin" derived from A. flavus toxin, was subsequently applied to the group of toxic compounds produced by this fungus.

Animal Species Susceptibility to Aflatoxins

Aflatoxins are acutely toxic for most animal species. Aflatoxin B_1 is the most toxic component of the aflatoxins and has been the most extensively studied for its lethal potency.

Following the outbreak of the Turkey 'X' disease, controlled feeding experiments were carried out with turkey poults, ducklings, and chickens. Early studies involving contaminated feeds suggested the duckling was the species the most susceptible to acute poisoning. The LD_{50} of day-old ducklings to aflatoxin B_1 is about 0.37 mg/kg (Carnaghan et al. 1963), which is a considerably smaller LD_{50} value than for the rat (1.0 mg/kg), and for the hamster (10.2 mg/kg) (Butler, 1964). However, later studies indicated that the dog, rabbit, and guinea pig have LD_{50} values of the same magnitude (0.5 mg/kg) as the duckling. The same observation is true for the rainbow trout (0.5 mg/kg) (Ashley et al. 1965). The LD_{50} values given were calculated from the mortality observed over seven-day periods.

In most species, death usually occurred in the first 72 hours after administration of the aflatoxin and necropsy at this stage revealed gross liver damage as the consistent pathological sign. Hemorrhage in the intestinal tract and peritoneal cavity and ascites was

occasionally seen in some species (Wogan, 1966). Butler (1964) found the principal histological change in rat liver was the development of a periportal zone of necrosis 3 to 4 days after dosing, with marked biliary proliferation. The latter lesions persisted after one month.

The relative lethal potencies of the four aflatoxins in the one-day-old duckling have been examined by Carnaghan et al. (1963). The oral seven-day LD_{50} values reported for each compound were: aflatoxin B_1, 18.2 µg; B_2, 84.8 µg; G_1, 39.2 µg; and G_2, 172.5 µg. All values in this instance were reported on a 50 g body weight basis. These values illustrate clearly the relationships of structural configuration to acute lethality (see Figure 1). Aflatoxin B_1 is most potent, followed by G_1, B_2, and G_2 in order of decreasing potency. The presence of the additional oxygen in the G compounds results in decreased activity by a factor of about 2, whereas the unsaturated compounds are approximately 4.5 times as potent as the dihydro derivatives.

Aspiration of spores of A. flavus into the respiratory tract failed to produce toxicity or lesions in ducks or chicks (Carnaghan and Allcroft, 1962). This experiment again established the fact that the fungal metabolite is the pathological agent and not the fungus as is the case with aspergillosis.

The general order of decreasing toxicity in poultry might be represented as follows: duckling, turkey, and chicken. For larger farm animals, the order of decreasing toxicity is: swine, cattle, horses, and sheep (Kraybill and Shimkin, 1964).

Of the larger farm animals, the pig appears to be the most susceptible, at least at the age of 3 to 12 weeks. Of the adults the pregnant sow is most commonly affected (Loosmore and Markson, 1961). Pigs are the only species that develop generalized clinical jaundice. With an increase in age the pigs become more resistant except with the pregnant sow mentioned above.

Calves are quite sensitive to aflatoxin, since exposure to aflatoxins leads to ductile proliferation and fibrosis in the liver. Calves from the age of 1 to 6 months are the most susceptible, but show increasing resistance with age (Kraybill and Shimkin, 1964). Feeding trials indicate that 3- to 4-year old heifers are clinically affected when they are continuously fed a ration containing 20 per cent toxic groundnut meal (Allcroft and Carnaghan, 1963a).

Cows fed toxic peanut meal excreted in the milk a toxic factor having a biological effect in ducklings (Allcroft and Carnaghan, 1963a). Pasteurization or drying of the milk did not reduce the toxicity of the milk for ducklings. Rennet precipitation of the milk proteins demonstrated that the aflatoxin resided in the cheese or paracaseinate fraction rather than in the whey. Allcroft and Carnaghan (1963b) maintained that none of their market-milk samples contained enough aflatoxin to give an effect on bioassay with ducklings. This may indicate that considerable dilution of possible toxins was experienced.

Sheep appear to be quite resistant to aflatoxin. Kraybill and Shimkin (1964) reported that three-month old sheep failed to demonstrate

any obvious effects other than a slight growth retardation after they were fed rations containing 20 per cent strongly toxic peanut meal for 19 months.

No information is available regarding mycotoxicoses in man caused by consumption of aflatoxin containing peanuts or other foods made from aflatoxin contaminated materials. Thus no evidence of a human hazard associated with the consumption of such products has been revealed. However, man is susceptible to mycotoxins as shown by the disease Alimentary Toxic Aleukia (ATA). An ATA outbreak occurred in Russia in the after-war years of 1942 to 1947 which was caused by the ingestion of over-wintered grain infected with a strain of Fusarium and its associated mycotoxin (Joffe, 1963). The morbidity rates in the populations affected were high, in some areas 10 per cent of the population being affected, resulting in many mortalities.

Chemical Nature of Aflatoxins

When extracts containing aflatoxins were viewed under ultraviolet light, a complex array of fluorescent compounds was generally present. The known aflatoxins comprise four of these fluorescent components. When chromatograms of extracts containing aflatoxins were viewed under ultraviolet light (3600 A^{o}), two strong bands were observed, one which was blue fluorescent and the other yellow-green fluorescent.

The most intense bands had R_f values (the ratio of the distance travelled by the component divided by the distance travelled by the solvent front) of 0.7 and 0.6 according to Sargeant et al. (1963). For these major bands, Wogan et al. (1963) reported R_f values of 0.75 and

0.69 for the blue fluorescent compounds and referred to them as compounds B_1 and B_2. The green fluorescent compounds with R_f values of 0.59 and 0.52 were reported as the G_1 and G_2 compounds. Nesbitt et al. (1962) separated the aflatoxin components B_1, B_2, G_1, and G_2 by countercurrent distribution in a solvent system of chloroform/carbon tetrachloride/water/methanol in the ratio (2 : 2.5 : 1 : 3) by volume.

The amounts and relative proportions of these four compounds present in culture extracts were variable, depending on such factors as mold strain, medium composition, and culture conditions. Typically, aflatoxins B_2 and G_2 were present in small relative amounts, whereas B_1 was usually present in the largest yield.

Structures based on interpretation of magnetic resonance spectral data were proposed for aflatoxins B_1 and G_1 in 1963 (Asao et al. 1963) and for B_2 (Chang et al. 1963, VanDorp et al. 1963) and G_2 shortly thereafter. The proposed structure of G_1 had been supported by X-ray crystallography (Cheung and Sim, 1964). These closely related compounds are highly substituted coumarins, and the presence of the furocoumarin configuration places them among a large group of naturally occurring compounds with many pharmacological activities (Soine, 1964). The chemical structures of aflatoxin B_1, B_2, G_1, and G_2 are shown on Figure 1.

The spectral characteristics of the aflatoxins have been determined by several investigators (Asao et al. 1963; Asao et al. 1965; De Iongh, 1962; and Hartley et al. 1963). Their ultraviolet absorption spectra are very similar, each showing maxima at 223, 265, and 363 mμ.

The molar extinction coefficients at the latter two peaks, however, demonstrate that B_1 and G_2 absorb more intensely than G_1 and B_2 at these two wavelengths. Because of the close similarities in structural configuration, the infrared absorption spectra of the four compounds are also very similar. The fluorescence emission maximum for B_1 and B_2 has been reported to be 425 mu, and that for G_1 and G_2 is 450 mu (Hartley et al. 1963). The intensity of light emission, however, varies greatly among the four compounds, a property of significance in the estimation of concentrations of the compounds by fluorescence techniques. The intensity also decreases with time of illumination, a factor affecting the assay procedures.

Carcinogenic Properties

In early investigations Lancaster et al. (1961) fed diets containing highly toxic peanut meals to rats. After six-month feedings of 20 per cent peanut meal (aflatoxin content not known) in a purified diet, 9 of 11 rats developed multiple liver tumors, and two of these developed lung metastases. This finding represented the first indication of the carcinogenic properties of aflatoxin-contaminated, toxic peanut meals, and has since been amply confirmed (Barnes and Butler, 1964; Butler and Barnes, 1964; Salmon and Newberne, 1963; Schoental, 1961). Subsequent investigations have been concerned with the demonstration that aflatoxins are responsible carcinogenic agents. The determination of dose, duration of exposure, and other conditions for tumor induction by aflatoxins are presently under investigation.

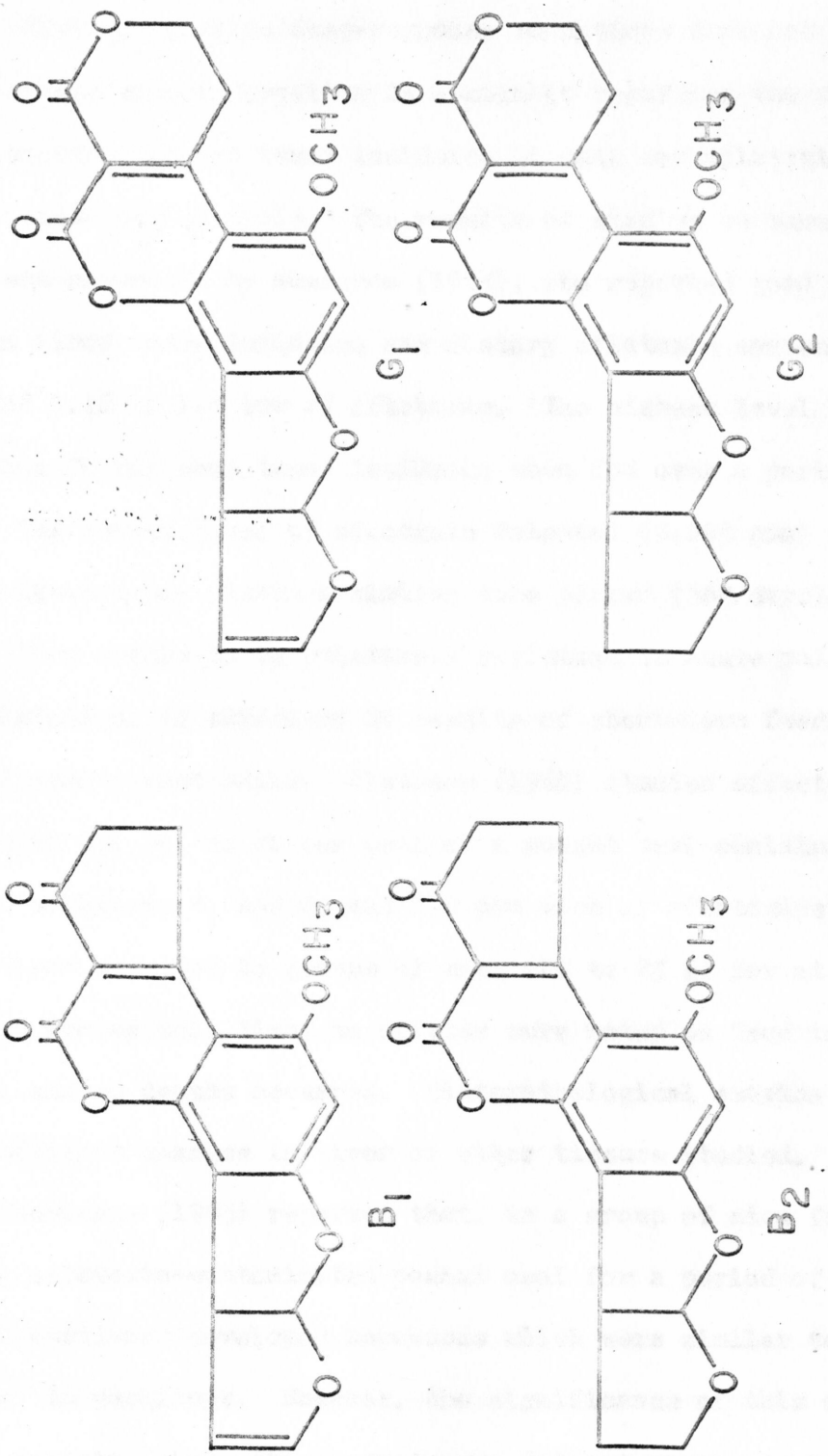

Figure I. Structures of the Aflatoxins

Although precise dose-response conditions have not yet been established, some information is available regarding the dose-response relationships between tumor incidence in rats and aflatoxin content of contaminated peanut meals. The results of studies on several such meals have been described by Newberne (1965), who reported good correlation between liver tumor incidence and dietary aflatoxin content over the range of 0.16 to 1.8 ppm of aflatoxin. The highest level resulted in more than 90 per cent tumor incidence when fed over a period of 370 days. The lowest level of aflatoxin detected (0.005 ppm) failed to induce liver tumor within a similar time period (384 days).

Mice appear to be relatively resistant to acute poisoning by the aflatoxins, as evidenced by results of short-term feeding of heavily contaminated peanut meals. Platonow (1964) studied effects of diets containing 15, 30, or 80 per cent of a peanut meal containing 4.5 ppm each of aflatoxin B_1 and G_1 and 0.6 ppm each of aflatoxins B_2 and G_2. These diets were fed to groups of mice (20 to 25 g) for at least three months. During this time, no effects were noted on feed intake or body weight, and no deaths occurred. Histopathological examination revealed no significant changes in liver or other tissues studied.

Newberne (1965) reported that, in a group of mice fed diets containing aflatoxin-contaminated peanut meal for a period of 16 months, 6 of 40 survivors developed hepatomas which were similar to those observed in ducklings. However, the significance of this observation is not certain, since the mouse tumors did not appear to be histologically malignant and metastatic lesions were not observed.

Recent experiments with purified aflatoxin preparations have indicated that continuous feeding is not required for hepatoma induction in rats. In the studies of Barnes and Butler (1964), rats were fed 1.75 ppm of aflatoxin (containing 80 per cent aflatoxin, with G_1 present in higher quantities than B_1) in the diet for 89 days, and were then returned to an aflatoxin free diet. All of three treated animals ultimately developed liver cancer after more than 300 days following withdrawal.

In similar experiments Wogan (1966) administered an unfractionated mixture of partially purified aflatoxins (approximately 30 per cent B_1, 20 per cent G_1) to rats by stomach tube. Each animal was treated daily for 30 days, and then was held without treatment for a further 10 months. Animals which received the highest dose (150 mg/day) had well-developed liver tumors 5 months after withdrawal of treatment. Even those rats receiving the lowest dose studied (15 mg/day) showed significant incidence of precancerous lesions at the same time interval. Wogan (1966) stated these lesions probably would have progressed to tumors over a longer period of time. This data indicates that continuous exposure to the compounds is not required for liver tumor induction.

On the basis of these preliminary data, it has been possible to estimate the effective dose of aflatoxin B_1 for the induction of liver tumors in rats. Butler (1965) estimated this dose to be in the order of 10 mg per day. When this value is compared with similar estimates for

other hepatocarcinogens, such as dimethylnitrosamine (750 mg/day) and butter yellow (9,000 mg/day), the much greater relative potency of the aflatoxin B_1 is readily apparent.

Recent studies (Ashley et al. 1964, Ashley et al. 1965, Sinnhuber et al. 1965) have suggested that the rainbow trout may be considerably more sensitive than the rat to the hepatocarcinogenic effects of the aflatoxins. These investigators have shown that rainbow trout develop liver tumors at significant incidence rates when fed purified diets containing only 0.5 to 2.0 µg of aflatoxin B_1 per kilogram (i.e., 0.5 to 2.0 ppb). The apparent sensitivity of this species had led to the recognition (Ashley et al. 1965) of the potential role of the aflatoxins as etiological agents in the so-called "trout hepatoma syndrome" (Halver, 1965; Hueper and Payne, 1961), which was observed in trout fed commercial feed meals containing aflatoxin contaminated cottonseed meal.

In a different test system, Dickens and Jones (1964) studied the effects of multiple subcutaneous injections of aflatoxins B_1 and G_1 in rats. A mixed preparation (about 38 per cent B_1 and 56 per cent G_1) of the compounds dissolved in peanut oil was administered to groups of rats twice weekly. One group received 50 mg at each injection, and the treatment was continued for 50 weeks; a second group received 500 mg at each injection for a period of only 8 weeks, after which treatment was discontinued. In the former group, six of six animals developed sarcomas or fibrosarcomas at the injection site within a 60-week period. At the higher dose level, five of five animals developed tumors within a 30-week period. These observations indicate that the compounds are

also carcinogenic for the subcutaneous tissues of the rat. Only one of the aflatoxin-treated animals at the higher dose level showed moderate liver lesions comparable to those resulting from oral administration.

From 1961 through 1964, Dickens (1964) studied the effects on the rat of the administration of a group of unsaturated lactones and found that a number of lactones are carcinogenic. The aflatoxins contain a similar type of unsaturated lactone structure to that found in a series of carcinogenic lactones. This may be one of the many possible explanations why the aflatoxins are potent carcinogenic agents.

Recently Sporn and co-workers (1966) suggested a mechanism for the carcinogenic effect of aflatoxin. They suggested that aflatoxin B_1 binds the deoxyribonucleic acid (DNA) and thus brings about a change in the metabolism of ribonucleic acid (RNA). This is considered to be a vital aspect of carcinogenesis caused by aflatoxins. This discovery also has great significance not only for aflatoxin toxicity but for cancer research as well.

As a consequence of the frequent opportunity for contamination of foodstuffs by mold metabolites such as aflatoxins, vital questions have arisen regarding the potential significance of these compounds in the etiology of several disease syndromes.

The profound carcinogenic potency of aflatoxin has implicated the possibility of their role in the etiology of primary liver cancer in man. According to Higginson (1964) there is at present no reliable information which would permit the association of aflatoxin with human hepatoma incidence. However, the incidence of this syndrome in man is

to a large extent restricted with regard to its geographic distribution within an area where food habits and food processing are relatively uncontrolled (Anonymous, 1963).

Production of Aflatoxins

For studies of the chemistry and toxicology of aflatoxins, it is desirable to have them available in sufficient quantity. Because their chemical synthesis has not yet been achieved, they must be produced by biological means. This may be readily done by growth of toxigenic strains of Aspergillus flavus on solid substrates such as ground peanuts (Codner et al. 1963), crushed wheat (Chang et al. 1963), and rice (Shotwell, 1966).

Their production in submerged culture or on semisynthetic media is preferable because of the ease of scale-up, simplicity of extraction and purification, and suitability of these media for studies on the physiology of aflatoxin production. The production of the aflatoxins on synthetic liquid media has been reported by De Iongh et al. (1962), Nesbitt et al. (1962), and Mateles and Adye (1965).

Protein or protein hydrolyzates will cause a marked increase in the production of toxin (Wogen et al. 1963). Good production of toxin can be produced by growing the fungus for 5 to 7 days at 27° C. on Czapeck's medium with added zinc sulfate (Kraybill and Shimkin, 1964). A typical mixture for fungal growth and toxin production was proposed by Mateles and Adye (1965) which utilizes glucose, ammonium nitrate, zinc sulfate, potassium dihydrogen phosphate, calcium chloride, and traces of other salts.

Pilot plant production of the aflatoxins is now carried out routinely. The fermentation of 100 liters of broth yields 10 grams of chloroform soluble material, of which 3 to 5 per cent consists of fluorescent compounds. From such a production batch, 300 mg of toxin was obtained for use in bioassay within a period of six days (Wogan et al. 1963). Rogovin (1966) developed a procedure for producing aflatoxins in Fernbach flasks containing 375 grams of rice cultured on shakers. By maintaining moisture at 30 per cent and incubating for five days at 28° C., a yield of aflatoxin B_1 greater than 1 gram per kg of rice was obtained.

Since protein or protein hydrolyzates will cause marked increases in the yield of toxin, Wogan et al. (1963) studied the influence of various amino acids on growth. Alanine, lysine, methionine, thyrosine, aspartic acid, histidine, arginine, cystine, glycine, isoleucine, hydroxyproline, ornithine, and phenylalanine had no effect. On the other hand, glutamic acid and proline stimulated production of the aflatoxin, whereas leucine, threonine, and tyrosine had a less pronounced effect. Just as the amino acid composition is critical for formation of toxin on various solid substrates and in liquid media, the ammonium compounds or ammonium nitrate also appears to be critical (Sargeant et al. 1963).

Animal products, rice, and corn appear to support toxin production better than peanuts. Soybeans or soya protein support weak production of the toxin. Protein sources such as wheat, oats, millet,

egg solids, and skim milk powder appear to support good growth and yields of toxin (Kraybill and Shimkin, 1964).

The yields of crystalline material, as reported in the literature, vary from one investigator to another, depending on the A. flavus strain, the culturing technique, the substrate used for growth, and the solvents used for extraction. Sargeant et al. (1963) obtained a yield of 20 mg of aflatoxin from 30 kg of toxic groundnut meal and left 40 mg unrecovered in the mother liquor. Wogan and co-workers (1963) reported a yield of 300 mg of aflatoxin from 100 kg of fermentation broth, or 40 mg of aflatoxin from growth of A. flavus on 250 grams of crushed wheat. Rogovin (1966) obtained yields of aflatoxin B_1 greater than 1 gram per kg when he grew A. flavus on rice.

Assay of Aflatoxins

I. Bioassays

In developing a simple bioassay method, complete extraction and satisfactory purification of the toxin by the use of suitable solvents and clean-up procedures are required. Several different bioassays have been developed in different laboratories around the world (Newberne et al. 1964, Verret et al. 1964).

Duckling. Ambrecht and Fitzhugh (1964) suggested that the day-old duckling was a suitable test animal.

In this method of assay, the sample under test was defatted and then extracted with the appropriate solvent. The solvent was then removed from the extract and the residue, after further purification by

appropriate procedures, was either suspended in water and administered by a polyethylene tube directly into the gizzard of day-old ducklings or incorporated into the diet. The basic pathologic response in ducklings associated with the administration of aflatoxin was a noninflammatory lesion which consists of a hepatic parenchymal necrosis and the proliferation of bile ductule cells. At the end of a week of daily administration of the toxin, the liver was pale or yellowish green in color and reduced in size. The severity of cytoplasmic vacuolization and hepatic cell damage appeared to change with time, but the proliferation of bile ductule cells progressed markedly. A careful histopathologic study has shown that the nature of the lesion was the same regardless of whether the ducklings were fed toxic contaminated meal or pure aflatoxins; variation occurred only in degree and intensity (Carnaghan et al. 1963). Meal contaminated with aflatoxin with as little as 30 ppb could be detected by this method. However, this method is relatively laborious, expensive, and time consuming, and requires experience in tissue pathology as well as both positive and negative controls which are essential in each group of assays.

Chick Embryo. The possibility of using chick embryos (Verrett et al. 1964) as a test organism for determining the toxicity has been investigated and found to be feasible. The injections of test solutions into fertile White Leghorn eggs were made before incubation, by either of two routes, the yolk or the air cell. The development of the embryos was observed for the full 21-day incubation period. The injection of extracts from toxin contaminated meal resulted in a toxic response

(LD_{50}) that correlated well with that obtained by injection of pure aflatoxin B_1 solutions at the same dosage levels. Meal contaminated with as little as 25 ppb aflatoxin was easily detected by this method. This method has the same disadvantages as the duckling assay.

Cell Culture. Several other biological systems can be employed to test for toxicity of the aflatoxins. For example, Juhasz and Greczi (1964) used different chick cell lines in cell culture. The assay by use of sensitive microorganisms was studied by Burmeister and Hesseltine (1966) who concluded their method was not sufficiently defined for use.

Bioassays are advantageous in that the aflatoxin does not need to be as highly purified as with chemical methods, if no other toxic factors are present, as discussed later under chemical methods of assay. However, the biological responses vary and give a great deal of ambiguity to the results. Much work still needs to be done to evaluate the degree of specificity and the practical significance of such biological systems. Therefore, the physical-chemical methods of assay of the aflatoxins were investigated for use in this study.

II. Chemical Assays

The isolation of aflatoxins was greatly facilitated by the discovery that the known aflatoxins B_1, B_2, G_1, and G_2 are strongly fluorescent in ultraviolet light with a maximum wavelength of 3600 A°. This property has provided a convenient means for monitoring isolation and purification procedures. The original investigations (Allcroft et al. 1961, Sargeant et al. 1961b) demonstrated that the compounds were

extractable with methanol. A variety of extraction procedures have since been developed for use with various natural products or mold cultures on natural substrates, particularly in connection with chemical assays of agricultural commodities for aflatoxin contamination. These include aqueous methanol (Campbell et al. 1964; Nesheim, 1964; Trager et al. 1964), aqueous acetone (Pons and Goldblatt, 1964), and a hexane-acetone-water azeotrope (Goldblatt, 1965).

In the production and isolation of quantities of aflatoxins from mold cultures on solid substrates, a convenient extraction and concentration procedure involves total extraction of the culture with chloroform and subsequent precipitation of the aflatoxins in petroleum ether (Asao et al. 1963, Asao et al. 1965). The aflatoxins produced in cultures on liquid media are almost quantitatively removed by partitioning into chloroform (Adye and Mateles, 1964).

Extracts produced by these procedures usually contain complex mixtures of fluorescent compounds, which are separable into their individual components by chromatographic techniques.

Resolution on filter paper is incomplete (Sargeant et al. 1961a) but is greatly improved by the application of thin layer chromatographic procedures. Although several such systems have been developed, including the use of alumina as the support medium (Broadbent et al. 1963), the conditions most widely used involve separation on silica gel plates with a solvent of 3 to 5 per cent methanol in chloroform (Asao et al. 1965; De Iongh et al. 1965; Nesheim, 1964).

Investigations of Chen and Friedman (1966) indicated that the aflatoxin components could be separated by development in 3 per cent methanol in chloroform and 5 per cent methanol in chloroform containing 0.5 per cent acetic acid. Good separation of aflatoxin components was also obtained by Strezleck and Kogan (1966). They used 9 per cent methanol in chloroform and developed the chromatograms at 40° F.

A number of methods for quantitative analysis of aflatoxin B_1 content have been developed. The most recent of these is a densiometric analysis (Shotwell, 1966) which gives a very good quantitation. A spectrophotometric analysis of aflatoxins was developed by Nabney and Nesbitt (1964). However, the most common method of estimation of aflatoxin B_1 content is by comparing the fluorescence on thin layer chromatograms of suitably prepared extracts to standards of known concentration.

Since aflatoxin B_1 is the most prevalent (Carnaghan et al. 1963; Verrett et al. 1964; Newberne et al. 1964) and also the most toxic of the aflatoxin components, a measure of the B_1 is used as the basis for a quantitative estimation of the four aflatoxins. The biological tests (discussed earlier) and the chemical tests have generally been in good agreement with each other. With modifications of the chemical tests which facilitate better separation of the components, the chemical assays are even more reliable.

None of these methods, either chemical or biological, provides a method of analysis of afaltoxins which would allow a worker to screen

a large number of suspected toxic cultures or contaminated foodstuffs rapidly.

Before attempting to develop such a method, the author has carefully reviewed all existing methods. One of these developed by Peterson (1966) at the Northern Utilization Research and Development Division, U.S.D.A., 1815 N. University St., Peoria, Illinois, has the potential of allowing a rapid, quantitative analysis of aflatoxin B_1.

The extraction procedure which he developed involved the extraction of a 25 gm sample of material with 150 ml of 25 per cent methanol in chloroform. This mixture was agitated five minutes using an ultrasonifier. The sonified solution was drawn off and filtered through a bed of anhydrous sodium sulfate. This procedure was then repeated two more times using 100 ml aliquots of 100 per cent chloroform.

The combined filtrates were dried using a water aspiration system. The flask samples were heated in a 60° C. water bath. Using this method he felt he could analyze 20 samples per day.

Starting with this method, the author has made modifications to improve the speed and reproducibility of the analyses.

This thesis is a summary of the work which the author has done to develop a rapid, quantitative method of determination of aflatoxin B_1, as well as studies to determine whether known aflatoxin producing strains of molds are capable of producing detectible quantities of aflatoxins on a variety of human foods, and studies to determine the optimum physiological conditions for the production of aflatoxin.

Table 1. Physical and Biological Properties of Aflatoxins

Aflatoxin	R_f [a]	Fluorescence emission maximum [b]	LD_{50} of 50 gm ducklings [c]	LD_{50} of chick embryo [d]
		mμ	μg	μg
B_1				
Mol. wt. 312 $C_{17}H_{12}O_6$	0.75	425	18.2	.048
B_2				
Mol. wt. 314 $C_{17}H_{14}O_6$	0.69	425	84.8	--
G_1				
Mol. wt. 328 $C_{17}H_{12}O_7$	0.59	450	39.2	0.8-1.0
G_2				
Mol. wt. 340 $C_{17}H_{14}O_7$	0.52	450	172.5	--

[a] Wogan et al. 1963.
[b] Hartley et al. 1964.
[c] Carnaghan et al. 1963.
[d] Verrett et al. 1964.

MATERIALS AND METHODS

Materials

Organisms

The microorganisms used in this study are known to be commonly occurring aflatoxin producing strains of molds which had been isolated from contaminated foodstuffs. Four strains of the genus Aspergillus, Aspergillus flavus and one species of the genus Penicillium used in this study were obtained, through the courtesy of Dr. C. W. Hesseltine, from the culture collection of the Northern Utilization Research and Development Division, U.S.D.A., 1815 N. University St., Peoria, Illinois, 61604:

Aspergillus flavus, Link, NRRL 2999
Aspergillus flavus, Link, NRRL A-13367
Aspergillus flavus, Link, NRRL 3000
Penicillium rubrum, Stoll, NRRL A-12701

In addition, the organism Aspergillus flavus, Link, ATTC 15517 was obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852.

I. Culture Maintenance. These organisms were carried in stock culture on potato dextrose agar slants. Transfers were made every seven days and the cultures were incubated at 30° C.

II. Confirmation of Purity of Cultures. The cultures were examined for contamination and confirmation of their identity by using the slide culture technique described by Ajello et al. (1963) and by preparing teased mounts mounted in lactophenol cotton blue. Examination

of the slide cultures and teased mounts microscopically (450 X) confirmed that pure cultures of organisms of the genus Aspergillus and Penicillium were present.

III. Confirmation of Toxicity of Cultures. Confirmation of the aflatoxin producing capacity of the cultures was performed by inoculating the organisms in pure culture into sterile Erlenmeyer flasks containing 50 gm of rice soaked with 20 ml of distilled water. Cultures were incubated for seven days at 32° C. and were then autoclaved at 121° C. for 15 minutes to kill the mold and to stop aflatoxin production.

The mixture of aflatoxins was extracted and assayed for aflatoxin B_1 content using the procedures described in the Methods section. All the cultures were tested when they were received and were confirmed to be capable of aflatoxin production under these conditions.

Extraction Equipment

(1) Burrell "Wrist Action" shaker - capable of holding eight 500 ml Erlenmeyer flasks.

(2) Erlenmeyer flasks - 500 ml.

(3) Funnel - 10 cm dia., long stem.

(4) Filter paper - Whatman #4, 18.5 cm. dia. circle.

(5) Vials - 4 dram capacity, fitted with polyethylene friction fit covers.

(6) Drying flasks - 500 ml capacity round bottom flasks with 25/40 ground glass joints.

(7) Water bath - set at 60° C.

(8) Glass wool - Pyrex.

(9) Rubber stoppers - #7, fitted with a protective polyethylene film.

Thin Layer Chromatography (TLC) Equipment

(1) Thin layer adjustable applicator set at 250 micron thickness, Model S-II (Brinkmann Instruments Inc., Westbury, New York.)

(2) Hamilton syringe, 10 and 50 µl, for accurate delivery of 1 - 40 µl.

(3) Long wave ultraviolet lamp (3600 A°), equipped with 100 Watt long wave flood bulb for observing low aflatoxin levels. Blak-Ray Model B-100A.

(4) Spotting template constructed with notches designed to hold the microliter syring 1.5 cm from the bottom of the plate, with serrations to give uniform spacing of 1.5 cm between spots.

(5) Thin layer chromatography tank, 5 x 20 x 20 cm, lined on the sides with heavy filter paper.

(6) Thin layer chromatographic plate - standard glass 20 x 20 cm, 3.7 mm thick (Brinkmann Instruments Inc., Westbury, New York).

(7) Desiccator for storage of TLC plates - 12 x 11 inch pressure cooker with exhaust outlets sealed. One-half pound of anhydrous calcium chloride added as desiccant.

(8) Dispatch oven - set at 94° C. to activate plates.

(9) Drying rack for thin layer plates - made by fastening two test tube racks together.

Other Equipment Used

(1) Bottles - 45 mm wide mouth with 140 ml capacity with metal screw caps. The bottles were fitted with filter paper strips which were saturated with water prior to incubation to control relative humidity.

(2) Humidity chamber - 8 x 12 inch metal container with removable cover filled with one-half inch of water to maintain a high humidity for cultures.

Aflatoxin Standards

A standard aflatoxin solution (SA 3-1) was obtained from the Southern Utilization Research and Development Division, U.S.D.A., P. O. Box 19687, New Orleans, Louisiana, 70119. This standard was prepared from pure crystalline aflatoxin B_1 and G_1. The standard contained:

Aflatoxin B_1 - 0.00060 micrograms per microliter

Aflatoxin G_1 - 0.00040 micrograms per microliter.

The standard was protected from light and was preserved by placing the 10 ml volumetric flasks containing the aflatoxins in a screw cap jar containing several ml of chloroform and storing in a freezer at -20° C. Before opening the jar, the temperature of the jar and the contained flasks was equilibrated to room temperature to prevent moisture condensation in the chloroform solution. Storage under these conditions minimized evaporation of the standard and oxidation of the aflatoxins.

Reagents

(1) Chloroform - A.C.S. grade.

(2) Methanol - A.C.S. grade.

(3) Sodium sulfate - A.C.S. grade, granular, anhydrous.

(4) Silica Gel CHR - Brinkmann Instruments Inc., Westbury, New York.

(5) Anhydrous calcium chloride.

(6) Extraction solvent - 25 per cent methanol : chloroform (V/V).

(7) Thin layer chromatography developing solvent - 3 per cent methanol : chloroform (V/V).

(8) Sodium lauryl sulfate - A.C.S. grade. 0.01 per cent solution (W/W) in distilled water.

Methods

Preparation of Food Substrates

The commercial food containers were opened using aseptic technique and triplicate 20 gm portions were weighed into sterile, wide-mouth bottles. After inoculation with the mold spores, the bottles were closed with metal screw caps fitted with filter paper inserted inside the caps. This filter paper was soaked aseptically with sterile water prior to inoculation to insure a high relative humidity over the substrate to minimize evaporation of the substrate moisture. Bottles containing the inoculated food were then placed in a humidity chamber to insure that there was little or no evaporation of substrate moisture.

The food samples were incubated for the length of time and the temperature specified in the experimental procedure and then were autoclaved to prevent release of viable spores into the atmosphere and to stop growth and toxin production at desired time intervals.

Inoculation of Foods with Mold Spores

Spore suspensions for inoculating foods were prepared by suspending the spores on a seven-day old potato dextrose agar slant culture in 4 ml of 0.01 per cent sodium lauryl sulfate. The spores were suspended by agitation using a sterile cotton swab. The spore suspensions were then either used directly for inoculum or were combined with other suspensions and diluted with sterile distilled water to obtain a spore suspension of sufficient concentration and volume to provide an adequate inoculum. The surface of the inoculated foods was distinctly colored green by the inoculum. However, no detectable amounts of aflatoxins were observed in the inoculated control foods when this concentration of spores was used.

Investigations on the applicability of an ultrasonifer in the extraction procedure described by Peterson (1966) were performed. Suspensions of 20 gm of presoaked rice in 150 ml of 25 per cent methanol: chloroform (V/V) in different shapes and sizes of separatory funnels and flasks were treated with a sonifier.

It was observed that agitation of the rice suspension during treatment with a 20KC sonifier was more of a swirling action rather than a violent disruption as anticipated.

Because of this, a number of other agitation methods capable of modification into a rapid extraction procedure were investigated. Use of a Waring blender gave good agitation in a short time (4 minutes), but the time involved in the rinsing and cleaning of the blender following each extraction, plus the danger of explosion (no explosion-proof blenders were available), ruled out this possibility.

Pons and Goldblatt (1965) reported the use of a Burrel Wrist Action shaker for the extraction of aflatoxin using 500 ml Erlenmeyer flasks and a one-half hour extraction. The possibility of adapting this method of agitation was investigated as the Burrel shaker has the capacity of extracting eight samples simultaneously. Good agitation of 20 gm of rice in 150 ml of 25 per cent methanol: chloroform (V/V) was observed in 500 ml Erlenmeyer flasks using this method.

Recovery experiments were performed designed to determine how many extractions using the Burrel shaker are necessary for complete removal of aflatoxin from a sample. A 20 gm sample of presoaked rice containing 60 ppb of aflatoxin B_1 was extracted using the procedures described later in the Materials and Methods section.

After agitation for one-half hour on a Burrel shaker, the aflatoxin containing suspension was filtered, and the rice residue was rinsed with 100 ml of 100 per cent chloroform. The combined filtrates were collected in a 500 ml round bottom flask.

The rice residue was then quantitatively transferred to a clean extraction flask and extracted as before. The suspension was filtered

and the rice residue was rinsed with 100 ml of 100 per cent chloroform, and the combined filtrates obtained were collected in a 500 ml round bottom flask.

The two filtrates obtained above were evaporated to dryness and resuspended in chloroform and analyzed using the thin layer chromatography (TLC) procedures described in the Methods section.

Complete removal of the aflatoxin was obtained with one extraction and one washing with 100 ml of 100 per cent chloroform, since the first extract was found to have the equivalent of 60 ppb of aflatoxin B_1. No aflatoxin could be detected in the second extraction filtrate.

Therefore, the author used the following method of extraction throughout this study.

Aflatoxin Extraction Procedure

(1) The autoclaved substrate was quantitatively transferred into a 500 ml Erlenmeyer flask. The bottle which contained the substrate was rinsed twice with 75 ml of a 25 per cent methanol:chloroform solution (V/V) and swirled to insure suspension of any toxin which might be present on the sides of the container. This suspension was then decanted into the 500 ml flask containing the substrate.

(2) The Erlenmeyer flask containing the substrate in the chloroform methanol solution was stoppered with a rubber stopper which was covered with a polyethylene film to protect the stopper from the solvent. The flask was placed on a Burrel shaker and the rate of agitation adjusted so the sample material collecting on the neck of

the flask was constantly washed back into the solvent. The flask was then agitated at such a rate for 30 minutes.

(3) After the flasks were shaken for 30 minutes, the flasks were removed and the contents decanted into a 100 mm ID funnel fitted with a 18.5 cm circle of Whatman #4 filter paper. The filtrate obtained was directly drained into another 100 mm funnel plugged with glass wool on which had been placed an anhydrous bed of solium sulfate (40 gm). The filtrates obtained in these filtrations were collected in a 500 ml round bottom flask.

The filtration was repeated twice using 100 ml aliquots of 100 per cent chloroform. The original extraction flask was rinsed each time to insure complete removal of the aflatoxin. The filtrates obtained from these two rinses were combined with the other filtrates in the 500 ml round bottom flask.

(4) The filtrate was placed on a 60° C. water bath and dried using an aspiration system. The residue obtained was resuspended in exactly 10 ml of 100 per cent chloroform. Care was taken to rinse the sides of the flask thoroughly. This 10 ml suspension was then transferred quantitatively to a stoppered vial and stored in a deep freeze (-20° C.) until thin layer analysis was performed on the sample.

Thin Layer Chromatography Assay Procedure

(1) Thin layer chromatographic plates were prepared as follows: 30 gm of silica gel GHR was shaken with 65 ml of distilled water for 45 seconds in a 250 ml stoppered Erlenmeyer flask and poured into a Brinkmann thin layer chromatographic applicator Model S-11 which had

been set for 0.25 mm (250 micron) thickness coating. The mixture was immediately coated on four 200 x 200 glass plates. The plates were allowed to air dry for 10 minutes, placed into a drying rack and dried in a Dispatch hot air oven for 4 hours at 95° C., and then stored in a desiccator over calcium chloride desiccant.

If the prepared plates had been stored more than 48 hours in the desiccator, the plates were reactivated for one hour at 95° C., and then allowed to cool to room temperature in the closed desiccator before samples were applied.

(2) Thin layer chromatographic plate developing tank preparation: A mixture of 150 ml of 3 per cent methanol in chloroform (v/v) was placed in a 5 x 20 x 20 cm chromatographic tank. The sides of the tank were lined with filter paper which dipped into the solvent system. The chamber was covered and allowed to stand for at least 20 minutes for solvent equilibration before the spotted plates were added for development.

(3) Spotting. The vials containing the sample extracts which were stored in a freezer were warmed to room temperature in the dark. Internal standards and samples were spotted by placing 2, 5, and 10 µl portions of the solution of the sample extract on a TLC plate and 1, 2, 3, and 4 µl of the aflatoxin standard solution along a line 1.5 cm from the bottom of the plate. On the same plate were spotted another 5 µl portion of the sample extract and directly on top of it 4 µl of the aflatoxin standard solution as an internal standard. The sample and the standard spots were spaced 1.5 cm apart.

For quantitative analysis, small tight spots are desirable. Accordingly, it was important that the sample standard spots be of the same size and no larger than 0.5 cm. The addition of the extract in 1-2 µl increments and having a fan or hair drier blowing on the spot to promote solvent evaporation aided in obtaining small uniform spots. Holding the microliter syring in an upright position aided in obtaining uniform round spots.

(4) Development of plate. Immediately after spotting, about 2 mm of the silica gel coating was removed from the sides of the plates. The plates were placed in an equilibrated chromatographic tank containing 3 per cent methanol:chloroform (V/V) and developed in total darkness. The plates were removed when the solvent front had travelled about 14 cm beyond the origin and the plates were allowed to dry in air for 10 minutes at room temperature in total darkness.

(5) Examination evaluation of plate. The plates were examined under a long wave ultraviolet light situated 30 cm from the plate in a darkened room. The examination sequence was first the 5 µl sample aliquot and then the 5 µl sample aliquot containing the internal standard to verify the presence or absence of a bluish fluorescent aflatoxin B_1 spot at a R_f of about 0.5 and a greenish fluorescent G_1 spot at R_f of about 0.4.

If aflatoxins B_1 or G_1 or both were present, the fluorescent intensity of the sample spots and standards were compared to select spots which most nearly matched in intensity. If necessary, interpolation of the sample spot was judged to be between two of the standard spots.

When the TLC analysis indicated the sample spots were too weakly fluorescent for reliable measurement, or if no aflatoxin could be detected in the sample spots, the sample extract was dried on a steam bath, resuspended in 5 ml of chloroform, and the assay was repeated using 5, 10, and 20 µl of the sample extract.

When the TLC assay indicated that the sample spots were too intensely fluorescent for reliable matching, the sample was dried and resuspended in a suitable volume of chloroform (15-20 ml). The assay was repeated using 2, 5, and 10 µl of the sample extract.

Calculations (Pons and Goldblatt, 1965)

$$\text{Parts per billion aflatoxin } B_1 = \frac{(V_s)\ (C_s)\ (S.D.)\ (1000)}{(w)\ \ (x)}$$

Where,

(V_s) = µl of aflatoxin standard in which the B_1 spot matches the sample spot B_1.

(C_s) = Concentration of aflatoxin B_1 in the standard aflatoxin solution; µg/µl.

(S.D.) = Volume to which sample extract is diluted for TLC analysis, in microliters.

(w) = Sample weight, in grams.

(x) = Volume of sample extract spotted, in microliters.

Safety Precautions

Little is known of the potential pathogenicity of the organisms studied or of the effects of aflatoxins in man. Because of this,

meticulous care was taken to avoid spillage of toxins, formation of mold spore aerosols, and safe disposal of contaminated equipment.

Before inoculations or transfers of cultures were made, an absorbent towel, well moistened with 2 per cent amphyl disinfectant, was placed on the work area. Pipettes and other contaminated discard materials were placed in an amphyl filled container after use. All cultures and materials used were autoclaved upon completion of laboratory manipulations.

If spillage of the toxin occurred, the area was washed with Chlorox bleach which is known to detoxify the toxin (Rogovin, 1966). The work area was examined in the dark using a long wave ultraviolet lamp to detect if any inadvertent and unnoticed spillage had occurred.

Protective clothing such as a laboratory coat or apron was worn during the process of handling the extracts. When manipulation of the toxic cultures and the extracts was completed, the worker's hands and arms were thoroughly washed with soap and water.

RESULTS AND DISCUSSION

Quantitation of Aflatoxin B_1 Recovery and Limits of Detection of Extraction Procedure

Aflatoxin recovery experiments were performed to determine the percentage of recovery of the extraction procedure described in Materials and Methods. The experimental recoveries of aflatoxin were determined by the addition of known amounts of a concentrated aflatoxin solution to samples of nonaflatoxin containing rice. The rice was then extracted and thin layer chromatography (TLC) analyses were performed. The percentage recoveries were calculated by dividing the concentration in parts per billion (ppb) of aflatoxin B_1 which was recovered by the concentration in ppb of aflatoxin which had been added. A preliminary dilution experiment was performed on the concentrated aflatoxin solution to determine what volumes of this solution, when diluted to the volume of the final extract, would have a concentration of aflatoxin B_1 which would allow TLC analyses at both high and low concentrations on a single TLC plate.

These volumes were added to 20 gm samples of nonaflatoxin containing rice which had been presoaked in 10 ml of water for 20 hours prior to addition of the aflatoxin.

The same volumes of the concentrated aflatoxin solutions that were added to rice were added to dilution tubes and suspended in a volume of chloroform which corresponds to the final volume of the resuspended extracts. These solutions were designated as the "reference

standards" because they were used to calculate the percentage of recovery.

Upon completion of extraction, preliminary TLC analyses were performed on the extracts. Equal volumes of the extract suspension and its corresponding reference standard were spotted and viewed under U.V. light. Visual comparisons of the fluorescence of aflatoxin B_1 of the two solutions on TLC plates gave an indication of the relative concentrations and, therefore, the recovery.

If the fluorescence of the extract appeared to equal that of the reference standard, it was again analyzed on a TLC plate to compare its fluorescence against the reference standard. Different volumes of the two solutions were spotted to allow improved estimation of the fluorescence of the solutions. If the intensity of the fluorescence was the same for various volumes of the extract compared to that of the reference standard, the per cent recovery was said to be 100 per cent. This comparison was subject to error of visual estimation of fluorescence.

However, if the fluorescence of the extract was less than that of the corresponding reference standard, TLC analyses were performed to determine the concentration of aflatoxin B_1 in the two solutions. TLC analyses were made on single plates to compare the fluorescence of both the extract and the reference standard with that of a primary standard (see Materials and Methods).

The concentrations of the two solutions were calculated by comparison of the fluorescence of a known primary standard (see Materials and Methods). The percentage recovery of the extraction procedure was

then calculated using the following formula:

$$\text{Percentage recovery} = \frac{\text{ppb of aflatoxin } B_1 \text{ recovered in extract}}{\text{ppb of aflatoxin } B_1 \text{ added as in reference standard}} \times 100$$

The results of this experiment of the percentage of recovery are shown in Table 2. At concentrations of 20 ppb and higher, the per cent recovery of aflatoxin B_1 was 100 per cent. At concentrations of 10, 5, and 1.5 ppb of aflatoxin B_1, the per cent recovery was 80 per cent, 60 per cent, and 0 per cent, respectively. At concentrations of aflatoxin B_1 greater than 20 ppb, the percentage of recovery is very high; losses of aflatoxin B_1 were at a level less than those detectable by TLC. At lower concentrations of aflatoxins, the recovery was less and less to the point where it was no longer discernible.

The smallest amount of aflatoxin B_1 on TLC which can be detected by visual examination under ultraviolet light is 0.000 5 ug (Chen and Friedman, 1966). The author's calculations were based on a primary standard containing 0.0006 ug/ul, thus the smallest quantity of aflatoxin B_1 that can be detected on TLC chromatograms using the primary standard used by the author if added in amounts no smaller than 1 ul is 0.0006 ug.

The final volume of the extracts themselves, therefore, limit the smallest amount of aflatoxin that can be detected. For example, if the final volume of the aflatoxin extract is 10 ml and the largest aliquot of the extract spotted is 20 ul, the limit of detection, using the formula for calculations and the primary standard described in Materials and Methods is 15 ppb. However, when this extract is resuspended in 5 ml and spotted, the limit of detection is 7.5 ppb.

Table 2. Percentage of Recovery of Aflatoxin B_1 from Rice Using the Standard Extraction Procedure

Aflatoxin B_1 added[a]	Aflatoxin B_1 recovered[b]	Per cent recovery of aflatoxin B_1[c]
ppb	ppb	%
360	360	100
300	300	100
240	240	100
180	180	100
120	120	100
60	60	100
20	20	100
10	8	80
5	3	60
1.5	0	0
0	0	0

a/ Aflatoxin B_1 added to 20 gm samples of presoaked rice, determined by TLC in reference to a primary standard.

b/ Aflatoxin B_1 concentration in extracts as analyzed by TLC in reference to a primary standard.

c/ These values were determined on TLC plates by visual comparison of fluorescence with two or more dilutions of standard.

Analysis systems used by other workers such as Chen and Friedman (1966) utilized much smaller aliquots, only 500 µl of chloroform to obtain a system which would allow detection of aflatoxin B_1 concentrations at a fraction of a ppb, for example, 0.02 ppb.

The author did not attempt to use such small aliquots because the opportunity for gross error resulting from inaccurate spotting and evaporation is too great. For example, 5 µl of a 20 gm sample of extract suspended in 500 µl of chloroform, an amount which could be easily lost through evaporation, would represent a large error in the calculation of concentration. Also, if dilution of the extract was needed because the concentration of aflatoxin was too high, the volume of aliquots removed, although small, must be included in the final calculation of concentration of the extract. This volume adjustment would give opportunity for experimental error.

Using this method, an investigator can quantitatively analyze more than 50 food samples in one day. This is much faster than the extraction procedures reported by Nesheim (1964) who developed a system capable of analyzing six samples a day and Peterson (1966) who could analyze 20 samples in one day.

The extraction procedure cannot be used for foods of high lipid content, e.g., bacon, without a preliminary extraction with hexane, since the procedure cannot separate the lipid material from the toxin. Therefore, the applicability of the extraction procedure is limited to defatted substances or substrates low in lipid content.

It is recognized that presoaked rice to which aflatoxin has been added does not represent a natural material where the toxin produced has had the opportunity to diffuse into the substrate or be present in the mold mycelium. Samples that contain naturally contaminated substrates with known levels of aflatoxin would be needed to authenticate the percentage of recovery of the extraction procedure.

Aflatoxin Production in Selected Human Foods

Seventeen human foods were studied to evaluate whether they would be suitable substrates for mold growth and mycotoxin production. The foods were tested for the presence of any ultraviolet fluorescent compounds as a preliminary test before the actual inoculation of the foods. The analyses of the 17 uninoculated foods showed no fluorescent substances.

In preliminary experiments, aflatoxin producing strains of fungi were used to inoculate peanut butter, bacon, American cheese, cold meat (Bologna), bread, and grape jelly. The inoculated foods were incubated at 4° C. and 30° C., which represent the approximate refrigeration and room storage temperatures.

The inoculated foods were incubated at the above temperatures and periodically checked for growth over a period of seven days. No visible sporulation or discoloration of the foods was seen, although the foods were adequately inoculated with visible amounts of spores from seven-day cultures of the test organisms.

At the end of a seven-day incubation period, the foods were autoclaved and subsequently extracted and assayed for aflatoxin production. No aflatoxin production was detectable.

It was thought at the time that two factors might be contributing to the lack of growth of the test organisms: (1) the foods contained preservatives, i.e., sodium propionate, which could exert an inhibitory effect on the cultures; (2) the moisture content of the inoculated foods may have been inadequate to allow growth and sporulation.

Subsequent experiments were designed to eliminate the possibility of a moisture shortage. Water was added directly to the inoculated foods or put into the bottles containing the inoculated substrates. The foods were incubated in a humidity chamber at 32° C., a temperature at which good sporulation of the cultures had been observed on potato dextrose slants.

The foods listed in Table 3 were inoculated and incubated in this manner. Visual examination of the foods at various time intervals indicated that certain foods were suitable substrates for both mold growth and sporulation as evidenced by the presence of spores and discoloration of the food.

It was observed that duplicates of some foods both gave and failed to give growth as was evidenced by sporulation when duplicate bottles of food were inoculated with identical quantities of the same culture and incubated under the same conditions.

Table 3. Maximum Aflatoxin B_1 Production on Selected Human Foods by Five Toxigenic Organisms[a/]

Food	Water added	Organism	Aflatoxin B_1 production
20 gm	ml		ppb
Bologna	3	A. flavus NRRC A-13367	60
Bread (white)	0	A. flavus ATCC 15517	300,000
Grape jelly	0	A. flavus NRRC A-13367	3,000
Cottage cheese	0	A. flavus NRRC 3000	1,800
Processed cheese	0	P. rubrum NRRC A-12701	1,500
American cheese	0	P. rubrum NRRC A-12701	15,000
Grated cheese	0	A. flavus NRRL A-13367	40
Whole black pepper	5	A. flavus NRRL A-13367	30,000
Baby food--beef	0	A. flavus NRRL A-13367	30
Baby food--lamb	0	A. flavus NRRL A-13367	40
Baby food-veg. and turkey	0	A. flavus NRRL A-13367	20
Baby food-strained eggs	0	A. flavus NRRL A-13367	400
Macaroni	10	A. flavus NRRL A-13367	200
Yeast cake	2	A. flavus NRRL A-13367	20
Mustard	0	A. flavus NRRL A-13367	0[b/]
Raisins	3	A. flavus NRRL A-13367	0
Dried apricot	5	A. flavus NRRL A-13367	0

[a/] Cultural conditions included incubation at 32° C., for seven days in a humidity chamber.

[b/] No aflatoxin B_1 detected with reference to a primary standard of 0.0006 ug aflatoxin B_1.

Some of the foods listed in Table 3 were observed to support growth of certain strains of the test organisms while failing to produce growth under identical conditions with others. In one experiment all five organisms were inoculated into jelly but only one strain, A. flavus NRRL A-13367, produced growth and aflatoxin. However, in later experiments other strains, A. flavus NRRL 2999, A. flavus NRRL 3000, and A. flavus ATCC 15517 were observed to grow and produce aflatoxins in jelly under the same experimental conditions. The significance of these observations as they relate to food spoilage and aflatoxin production will have to await further investigations.

The foods were autoclaved after a seven-day incubation period, extracted using the standard procedure, and analyzed by the TLC method (see Table 3).

These results indicate that of the 17 foods tested under the conditions of the experiment 14 were capable of acting as substrates for aflatoxin formation. The maximum yield of aflatoxin B_1 was produced on bread (300,000 ppb); lower yields were produced on other foods (see Table 3).

No aflatoxin production was observed in foods which did not support visible growth. It was observed different foods gave production of aflatoxin B_1 at different levels with different test organisms (see Table 3).

Certain foods were not suitable substrates for supporting visible growth or aflatoxin production including dried apricot, mustard, and raisins. One can speculate that the presence of sulfur dioxide in dried

apricot (an antifungal agent) and possibly the presence of numerous spices in mustard was inhibitory to mold growth. Raisins apparently were not suitable substrates for mold growth under the conditions tested.

In an attempt to set a rating scale for toxicity of animal foodstuffs, Raymond (1966) placed the toxicity of aflatoxin B_1 in foods into the following categories of toxicity: very high, more than 1 ppm; high, 0.25 to 1.0 ppm; medium, 0.05 to 0.25 ppm; low or negative, less than 0.05 ppm.

At the present, there are no pharmacological data available indicating a safe level of aflatoxin in the diet of man. The aflatoxins are known to be carcinogenic agents; therefore, no tolerance can be set for their presence in human diets. At present, the Federal Food and Drug Administration does not permit any detectable aflatoxin in foods shipped in interstate commerce.

Experimental results indicated that the moisture content of the food is extremely important with respect to mold invasion and aflatoxin production, and is perhaps the easiest method of controlling mold growth and subsequent aflatoxin production. According to Austwick and Ayerst (1963), mold growth occurs very slowly, if at all, when moisture content is below 9 per cent.

It should be emphasized, however, that uneven distribution of moisture on a substrate may result in mold growth even though the average moisture content of the food appears to be at a safe level. A mold produces moisture as it grows, so once fungal growth has started on

an excessively moist portion of a food, the moisture content of the surrounding food also increases and further proliferation may proceed regardless of the average moisture content.

The potential hazard of a situation of this nature is demonstrated in the following example. If an individual peanut was found to have an aflatoxin B_1 content of 300,000 ppb (a value which has been observed), this peanut when mixed with 1000 peanuts which had no aflatoxin would give a mixture with an aflatoxin concentration of 300 ppb. This represents a potential toxicological hazard and one which could easily occur.

The growth that occurred on macaroni and whole black pepper upon the addition of sterile water demonstrated the potential hazard of improperly stored human foods. The foods, upon incubation at room temperature for seven days, produced visible fungal growth and sporulation. The foods then themselves carry viable spores which, when exposed to favorable conditions, germinate and grow. This becomes a problem of both spoilage and of toxin production if toxic organisms are part of the flora.

Therefore, it can be concluded that a wide range of human foods are subject to fungal invasion and are capable of supporting aflatoxin production. Whether a food produces toxin depends on the organism, the available moisture, the nature of the food, and presence of inhibitors.

Note should be made of the observed appearance and odor of the inoculated foods after a seven-day incubation period. The discoloration and the pungent odor of the grossly contaminated foods seems to the

author to prohibit the human consumption of heavily contaminated foods.

Information which would furnish some basis of assessment of the potential role of aflatoxin in an authentic human diet (one not grossly contaminated) is urgently needed. For example, a study of trimmed mold contaminated foods is needed to determine if aflatoxin is present in the apparently normal food. These studies would provide useful information for the development of procedures for the safe processing of contaminated foods. Information is also needed on the prolonged effects of the intake of very low levels of these aflatoxins which would not be in great enough concentration to express themselves in the form of acute toxicity.

Further investigations on the subject of mycotoxin production on human foods under a variety of physiological conditions are needed. Molds are capable of growth under a number of adverse physiological conditions. For example, molds are capable of growth at temperatures below 0° C. and up to 60° C. (Ainsworth and Sussman, 1965). Joffe (1963) reported that the mycotoxin causing Alimentary Toxic Aleukia in man is produced at -4° C. Obviously, a wide range of frozen and cooked foods is susceptible to mold invasion and subsequent mycotoxin production. Mold growth can occur with less than 1 per cent oxygen (Semeniuk, 1966), implying the possibility of growth in canned and sealed foods.

Aflatoxins are extremely stable compounds so their removal or destruction during the course of food processing is very difficult. To bring about their thermal decomposition, it would be necessary to raise the food temperature to 300° C. or above (Fischbach and Campbell, 1965).

Studies on methods for removal of aflatoxins from foods on a commercial basis by enzymes (Ceigler, 1966) and by solvent extractions (Vix, 1965) are in process. To date, the potential utility of these and other detoxification processes are limited.

Effect of Temperature, pH, and Moisture on Sporulation and Aflatoxin Formation

Experiments were designed to determine the effect of varied physiological conditions of temperature, pH, and moisture on sporulation and on production of the aflatoxins. A known aflatoxin producing organism, A. flavus ATCC 15517, used in these studies was grown on a rice substrate.

Twenty gram samples of the rice in 250 ml Erlenmeyer flasks fitted with cotton stoppers were inoculated with spores suspended in a 0.01 per cent sodium lauryl sulfate solution from a seven-day culture of the organism.

As listed in Table 4, five incubation temperatures, 4°, 23°, 32°, 37°, and 45° C.; five pH values, 4.5, 5.0, 5.9, 6.9, and 8.0; and five concentrations of added moisture, 13, 20, 33, 42, and 50 per cent by W/W were studied. These three variables were used to investigate all the possible combinations of physiological conditions for the test organism.

The temperature of the cultures was controlled by use of constant temperature incubators, the initial pH was controlled by the use of Sornesen's buffer, and the initial moisture content of the substrates was controlled by the amount of the added buffer.

At the two extremes of temperature, 4° and 45° C., no growth of the mold was detectable either by the presence of spore formation or by discoloration of the rice within a seven-day incubation period.

The optimum temperature for growth of this culture was 32° C. based on the amount of sporulation. Lesser amounts of sporulation were observed at 37° and 23° C. (see Table 5). The cultures incubated at 4° and 45° C. did not yield any detectable aflatoxin. At the optimum temperature for aflatoxin production, 23° C., aflatoxin production far exceeded the yield of production at the other experimental temperatures. In contrast, little aflatoxin was produced at 37° C. since only four cultures of a possible 25 produced aflatoxin (see Table 8). Cultures incubated at 32° C. produced toxin but at a level 100 times less than that produced at 23° C. (see Table 8).

It should be noted that sporulation of a culture is not necessarily an index of aflatoxin production. For example, at the temperatures 37° and 32° C. the cultures produced luxuriant sporulation compared to sporulation at 23° C. However, at 23° C. very high yields of aflatoxin were produced compared to 37° C. and 32° C. (see Figure 2). Therefore, estimation of possible food toxicity by the amount of visible sporulation could result in gross error.

The optimum moisture content for growth based on the presence of visible sporulation was observed with 15 ml of buffer added per 20 gm of rice or 43 per cent added moisture. Lesser amounts of sporulation were observed with 10, 20, 5, and 3 ml of added water. These values correspond to 33, 50, 20, and 13 per cent moisture (see Table 6).

Table 4. Selected Physiological Conditions of Temperature, pH, and Moisture Studied with Aspergillus flavus ATCC 15517 Grown on Rice

Temperature of incubation	pH (Initial buffered)	Per cent moisture added (W/W)
° C.		
4	4.5	13
23	5	20
32	5.9	33
37	6.9	42
45	8	50

Table 5. Effect of Temperature on Sporulation of Aspergillus flavus ATCC 15517 Grown on Rice[a]

Temperature of incubation	Amount of sporulation[b]
° C.	
4	0
23	+++
32	+++++
37	++++
45	0

[a] Incubation time was seven days.
[b] Sporulation was rated from zero to a maximum of five.

However, the optimum moisture content for aflatoxin production was 5 ml added to 20 gm or rice (20 per cent moisture). Lesser amounts of aflatoxin were produced with 33, 42, 50, and 13 per cent moisture.

Therefore, the amount of moisture which permits optimum sporulation of a culture does not necessarily permit maximum aflatoxin production.

The optimum initial pH for growth of the cultures, based on sporulation, is pH 6.9 at 37° and 32° C. At 23° C. the optimum initial pH for maximum sporulation of the culture is pH 5 (see Table 7).

However, the optimum initial pH for the production of aflatoxin at 32° C. is pH 5 and for 23° C. pH 8 (see Figure 2). It must be concluded the pH of the substrate plays a role at different temperatures in its stimulation or repression of aflatoxin production. Further work along these lines is needed with other substrates and organisms.

According to Frazier (1958) the most important environmental factors influencing mold growth are temperature and moisture. The optimum temperature for mold growth varies with the strain of the mold. For example, when corn was stored at a relative humidity of 80 per cent (corresponding to a moisture content of about 9 to 16 per cent), a species of Penicillium was predominant at 25° C., A. flavus at 30° C., A. glaucus at 35° C., and a species of Mucor at 45° C. (Forgacs, 1962).

The moisture requirements of an organism are affected by variations in temperature, substrate, and pH (Frazier, 1958). The moisture available to an organism is expressed in terms of water activity (A_w) which is the vapor pressure of the solution (substrate) divided by the vapor pressure of the solvent (water).

Table 6. Effect of Initial Moisture on Sporulation[a] of *Aspergillus flavus* ATCC 15517 Grown on Rice at Selected Temperatures

Temperature of incubation, °C.	Per cent water added				
	13	20	33	42	50
4	0	0	0	0	0
23	+	++	+++	+++++	++++
32	+	++++	+++++	+++	++
37	+	+++	++++	+++++	++
45	0	0	0	0	0

[a] Sporulation was rated from zero to a maximum of five.

Table 7. Effect of Initial pH on Sporulation[a] of *Aspergillus flavus* ATCC 15517 on Rice at Selected pH Values

Temperature of incubation, °C.	Initial pH				
	4.5	5	5.9	6.9	8
4	0	0	0	0	0
25	++++	+++++	+++	++	+
32	++	+	+++	+++++	++++
37	++	+	+++	++++	+++++
45	0	0	0	0	0

[a] Sporulation was rated from zero to a maximum of five.

Table 8. Aflatoxin B_1 Production by Aspergillus flavus ATCC 15517 Grown on Rice with Varied Physiological Conditions

pH	Moisture added	Temperature of incubation 23° C.	32° C.	37° C.
Initial	%	ppb Aflatoxin B_1	ppb Aflatoxin B_1	ppb Aflatoxin B_1
8	3	30,000	4,500	0[a]
8	5	300,000	450	600
8	10	150,000	300	600
8	15	150,000	1,500	0
8	20	60,000	600	0
6.9	3	30,000	25,000	0
6.9	5	450,000	1,500	0
6.9	10	60,000	600	0
6.9	15	60,000	600	0
6.9	20	30,000	300	0
5.9	3	30,000	600	600
5.9	5	300,000	30,000	0
5.9	10	90,000	3,000	0
5.9	15	60,000	600	0
5.9	20	30,000	600	0
5	3	60,000	7,500	0
5	5	450,000	25,000	0
5	10	300,000	600	0
5	15	90,000	1,500	0
5	20	30,000	1,500	0
4.5	3	30,000	3,000	0
4.5	5	450,000	0	0
4.5	10	450,000	300	0
4.5	15	90,000	300	300
4.5	20	30,000	600	0

[a] No aflatoxin B_1 detected in reference to a primary standard of 0.0006 ug of aflatoxin B_1.

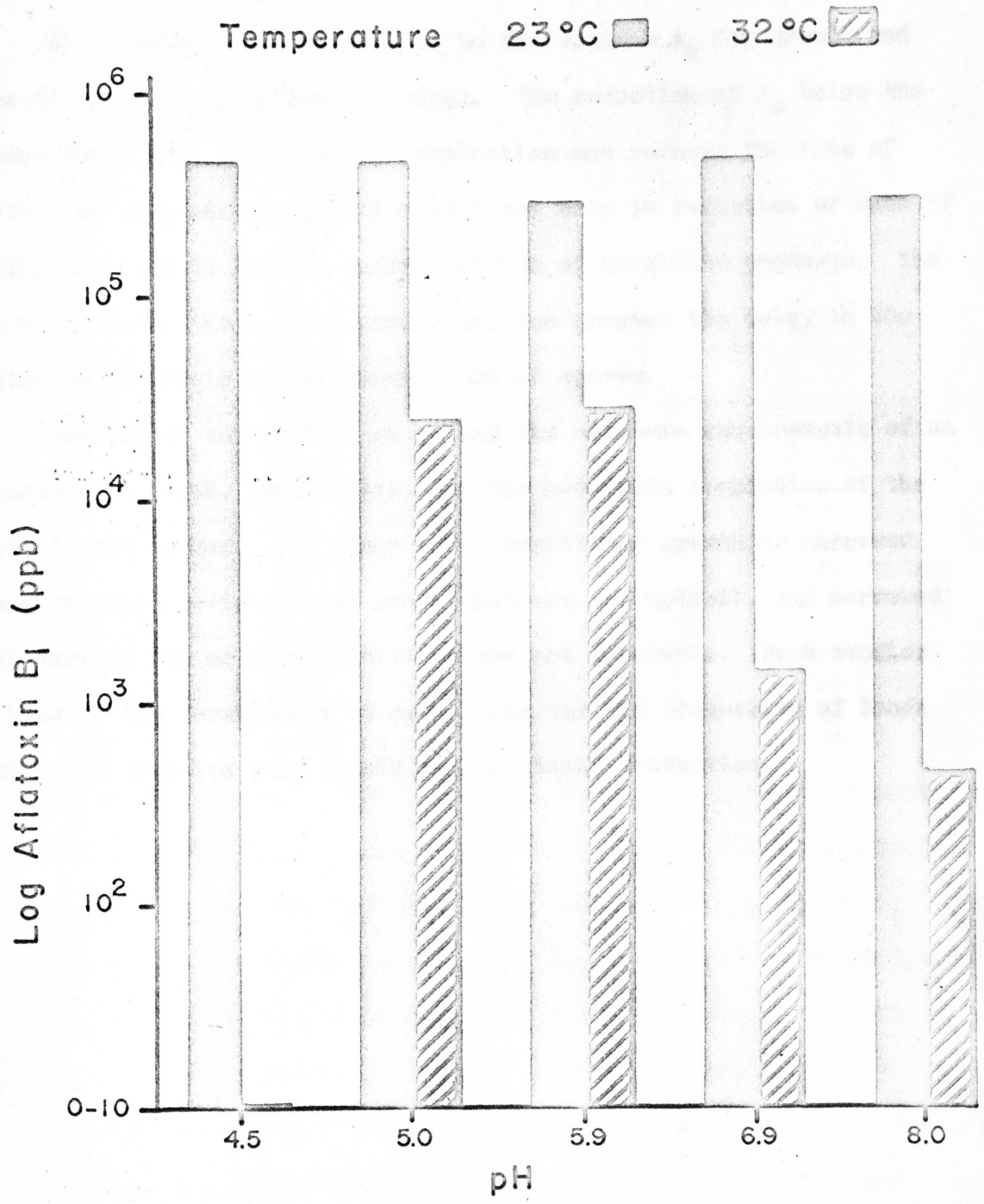

Figure 2. Effect of Temperature and pH on Aflatoxin B_1 Formation by A. flavus ATCC 15517 on Rice at 20 Per Cent Moisture in Seven Days

Molds differ considerably as to the optimum A_w for growth and range of A_w for germination of spores. The reduction of A_w below the optimum for a mold delays spore germination and reduces the rate of growth. An unfavorable A_w will result not only in reduction of rate of growth, but also in lowered maximum yields of metabolic products. The more unfavorable the A_w of a substrate, the greater the delay in the initiation of growth or the germination of spores.

Studies of the factors affecting the moisture requirements of an organism such as pH, temperature, and the nutritive properties of the substrate are needed. The range of A_w permitting growth is narrowed if any of these environmental conditions are not optimal, and narrowed still more if two or more conditions are not favorable. Such studies may lead to the development of procedures for the processing of foods which would minimize mold growth and aflatoxin production.

SUMMARY

A rapid modified procedure for the extraction of aflatoxins from low fat substrates was developed which gave quantitative recovery of aflatoxin B_1 down to 10 ppb. Using this method, an investigator can analyze 50 samples in one day.

Of 17 human foods studied, 14 foods such as cheeses, bread, jelly, and baby foods were found to be capable of supporting mold growth and the production of aflatoxin. Whether a food will support growth of a fungus and the amount of aflatoxin produced depends on (1) the strain of the fungus, (2) the food on which it grows, (3) the moisture content of the food, and (4) the presence of inhibitors in the food.

Varied physiological conditions of temperature, moisture, and pH were studied to determine the maximum and minimum production of aflatoxin by A. flavus ATCC 15517 grown on rice. The optimum temperature for aflatoxin production was 23° C. No growth or aflatoxin production was obtained at 4° and 45° C. after incubation for seven days.

The initial moisture of a food in the range of 13 to 50 per cent has a role in the production of aflatoxin and sporulation. Optimum sporulation of the cultures was obtained with 43 per cent moisture. However, the optimum moisture for aflatoxin production was 20 per cent. The optimum initial pH for the range of 4.5 to 8.0, varies with the temperature of incubation and the percentage of moisture of the substrate. The optimum production of aflatoxin at 32° C. was at an initial pH of 5.0; and at 23° C. it was at pH 8.0.

CONCLUSIONS

(1) A rapid quantatitive extraction procedure was developed for the removal of aflatoxins from low fat foods.

(2) Fourteen of 17 commercial human foods which were inoculated with aflatoxin producing strains of Aspergillus flavus and Penicillium rubrum were suitable substrates for sporulation and aflatoxin production.

(3) The suitability of a food for sporulation and aflatoxin production depends on the moisture content of the food, the presence of inhibitors, the nature of the food, the temperature of incubation, and the strain of the mold.

(4) Aflatoxin B_1 production on the human foods tested ranged from 300,000 ppb on bread to 0 ppb on dried apricots, mustard, and raisins.

(5) There is little danger of human consumption of grossly contaminated foods because of their pungent odor and discolorization.

(6) The mold A. flavus ATCC 15517 gave maximum production of aflatoxin B_1 when grown on rice at 23° C. (450,000 ppb) and smaller amounts at 32° C. (30,000 ppb) and 37° C. (600 ppb). No sporulation or aflatoxin was produced with this mold when grown at 4° and 45° C. over a seven-day incubation period.

(7) There is no direct correlation between the amount of sporulation and aflatoxin production.

(8) The initial pH of a substrate for the optimum production of aflatoxin varies with the temperature of incubation and the moisture content of the substrate.

Foods consumed by man which have been contaminated with aflatoxin represent potential substrates or carriers for toxic and carcinogenic agents, and represent an environmental source for the possible mediation of cancer through diet. Further work is needed to assess the public health hazard of aflatoxins in human diets, particularly in slightly contaminated foods.

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